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Chronic Treatment with Either Dexfenfluramine or Sibutramine in Diet-Switched Diet-Induced Obese Mice

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Dexfenfluramine (DEX) and sibutramine (SIB) are effective antiobesity agents. Their effects on weight control and hormone profile have not been previously studied in diet-switched diet-induced obese (DIO) mice, in which treatment is initiated upon cessation of a low-fat diet and resumption of a high-fat diet. Furthermore, their effects on circulating ghrelin in obese humans or in animal models of obesity have not yet been reported. Male C57Bl/6J DIO mice after 16 wk on a high-fat diet (HF, 60 kcal% fat) were switched to a low-fat diet (LF, 10 kcal% fat) for 50 d. HF diet resumed concurrently with treatment for 28 d with DEX 3 and 10 mg/kg, twice a day (BID); SIB 5 mg/kg BID; or vehicle. Rapid weight regain ensued in vehicle-treated DIO mice. DEX or SIB treatment significantly blunted the body weight gain. Caloric intake was decreased acutely by DEX or SIB vs vehicle during the first 2 d treatment, but returned to control after 5 d. At the end of study, epididymal fat weight and whole body fat mass determined by DEXA scan were decreased by DEX 10 mg/kg, and whole body lean mass decreased with DEX 3 mg/kg treatment. Circulating ghrelin on d 28 was increased with either DEX 3 or 10 mg/kg treatment, while growth hormone and insulin were decreased. Leptin was also decreased in the DEX 10 mg/kg group. SIB did not significantly affect fat mass, ghrelin, growth hormone, insulin, or leptin. Mice chronically fed LF diet maintained a lower caloric intake, gained less weight and fat mass than diet-switched mice, and had higher ghrelin and lower insulin and leptin. In summary, weight regain in diet-switched DIO mice is delayed with either DEX or SIB treatment. DEX treatment of diet-switched DIO mice decreased growth hormone, insulin, leptin, fat mass, lean mass, and increased ghrelin, while SIB only decreased body weight.

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Introduction

Obesity is a colossal public health and medical problem worldwide. According to data from the 1999–2000 National Health and Nutrition Examination Survey (NHANES), nearly two-thirds of adults in the United States are overweight, and more than 30% are obese *(1)*. Obesity is a risk factor for diabetes, heart disease, stroke, hypertension, osteoarthritis, and some forms of cancer. Obesity is related to a sedentary lifestyle and an energy-rich diet, but long-term maintenance of body weight with caloric restriction and exercise alone is often not adequate. Several drugs have been developed for the treatment of obesity. Currently approved drugs in the U.S. for chronic treatment of obesity are sibutramine (SIB) and orlistat. SIB is a mixed norepinephrine and serotonin reuptake inhibitor *(2)* that decreases appetite and increases resting energy expenditure. Orlistat is an intestinal lipase inhibitor that inhibits fat absorption. Like SIB, dexfenfluramine (DEX) is a serotonin reuptake blocker. However, it is also a serotonin releaser and serotonin receptor agonist *(2)*. The once popular combination of fenfluramine or DEX plus phentermine (fen-phen) was withdrawn due to cardiac valvulopathy, an effect that was attributed to the fenfluramine component *(3)*. Phentermine is still widely used, but its long-term efficacy as monotherapy is questionable. Fenfluramine has serotonin 5-HT2B agonist activity, and stimulation of 5-HT2B receptors in the heart may be responsible for the cardiac valvulopathy *(4)*. The 5-HT2C serotonin receptor subtype present on proopiomelanocortin (POMC) neurons in the hypothalamus mediates the anorexic effects of fenfluramine *(5)*.

One dilemma with chronic treatment of obesity is the tendency for individuals to regain lost weight quickly, possibly due to resumption of sedentary lifestyle, a calorie-dense diet, and cessation of exercise and pharmacotherapy. Mice placed on a HF diet gain excess body weight, mainly due to deposition of excess fat in adipose tissue. When these mice are switched to a LF diet, they lose weight, but the weight is quickly regained when they are switched back to a HF diet

(6). In this study, we initiated drug treatment at the same time that HF diet was reintroduced. We believe this diet-switched DIO mouse model is a rigorous, important tool for evaluation of the ability of test agents to retard or prevent the reemergence of the obesity phenotype and to assess the potential for long-term efficacy. SIB was compared with DEX for effects on body weight, food intake, fat/lean mass, and changes in several circulating hormones (insulin, leptin, ghrelin, and growth hormone) associated with the efficacy profiles of these two agents. This article summarizes a novel approach to the evaluation of pharmacotherapeutic agents, in which treatment is initiated at the same time a HF diet is reintroduced. The primary objective of this study was to determine whether SIB or DEX will prevent or retard body weight gain in this model, because either agent induces weight loss in obese mice. Prevention of weight gain was predicted to increase circulating ghrelin and growth hormone, while weight regain is expected to suppress both.

Results

DIO mice ($n = 80$) and age-matched lean mice ($n = 24$) on HF or LF diets for 16 wk weighed 42.59 ± .52 g and 30.56 ± .46 g, respectively ($p < 0.0001$ by t-test). The DIO mice were switched from HF to LF diet for 50 d, resulting in loss of body weight by d 0, to 39.26 ± 0.44 g, $n = 74$. On d 0, mice always on LF diet weighed 35.57 ± .65 g, $n = 18$.

A prompt weight regain (8.41 ± .63 g over 28 d) was observed in vehicle-treated mice switched back to HF diet (Fig. 1A). Significant overall differences in body weight were detected over time, and among treatment groups ($p < 0.0001$), by two way ANOVA with repeated measures, followed by comparisons of each treatment to HF–LF–HF vehicle group at each time point indicated by overall significant differences, using Bonferroni's post test. The weight gain associated with LF to HF diet switch was slowed significantly by DEX (3 mg/kg, 4.87 ± 1.01 g, –42%; 10 mg/kg, 3.54 ± 0.48 g, –58%) or SIB (5.68 ± 0.73 g, –32%), but after 28 d treatment body weight was maintained in DIO mice continued on LF diet (–1.59 ± 0.90 g, –119%).

Vehicle treated mice maintained on LF diet had a significantly lower rate of caloric intake throughout most of the 28 d treatment period than mice on HF diet (Fig. 1B, $p < 0.001$). However, mice maintained on LF diet ate significantly more between d 0 and 1 than those switched to HF diet. The rate of caloric intake was significantly decreased starting at 1 d of treatment with DEX at 3 or 10 mg/kg, or with SIB 5 mg/kg, relative to HF vehicle-treated mice. Only the lower 3 mg/kg dose of DEX significantly decreased rate of caloric intake beyond d 2 ($p < 0.05$, d 21 only), relative to HF vehicle control mice.

Vehicle-treated mice fed LF diet had significantly higher total plasma ghrelin (Fig. 2) than mice fed HF diet ($p < 0.01$). DEX 3 mg/kg and 10 mg/kg, significantly increased ghrelin, HF diet fed mice treated with SIB had plasma ghrelin similar to vehicle-treated mice on HF diet.

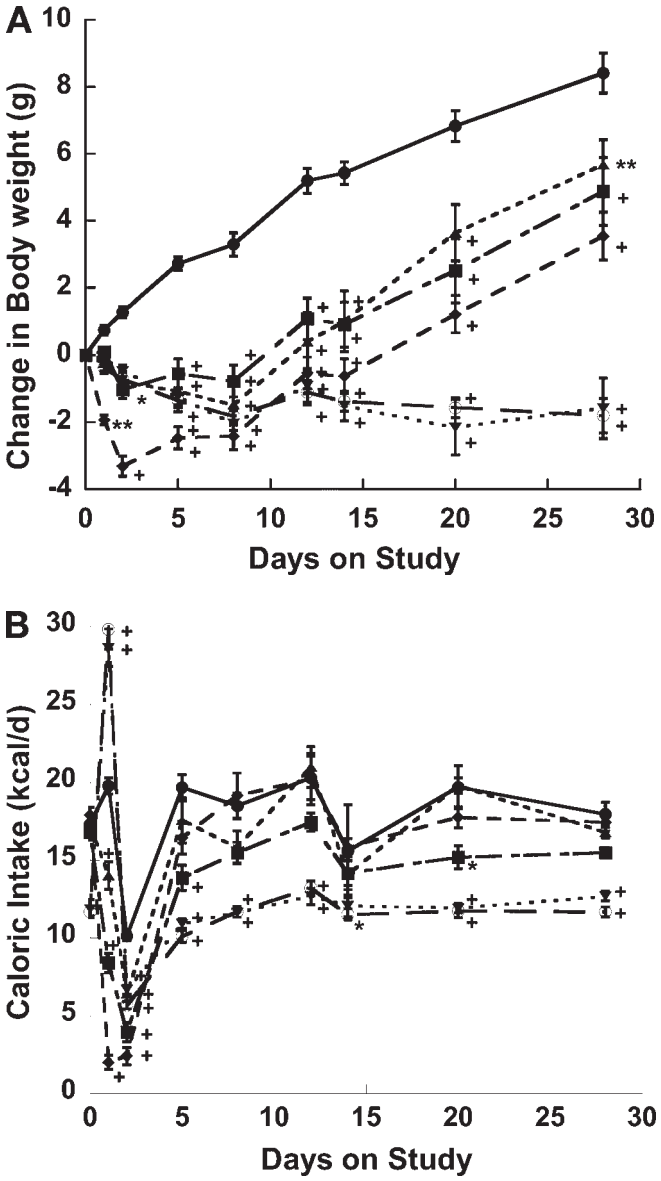

Fig. 1. Effects of DEX and SIB treatment on body weight and caloric intake in diet-switched DIO mice. (**A**) Changes in body weight from onset of treatment. (**B**) Caloric intake in diet-switched DIO mice. Treatment groups: ●, high–low–high vehicle treated mice; ■, DEX 3 mg/kg, po BID; ◆, DEX 10 mg/kg, po BID; ▲, SIB 5 mg/kg, po BID; ▼, high low vehicle; ○, always LF vehicle. Statistically significant differences by Bonferroni's test from time-matched high–low–high vehicle treated group are indicated: $^{+}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; $n = 12$/group.

Plasma GH in vehicle-treated mice fed LF diet was not significantly different DIO mice fed HF diet, after 28 d of treatment (Fig. 3). DEX at 10 mg/kg significantly decreased GH relative to vehicle-treated mice on HF diet opposite to the effect on ghrelin. HF diet fed mice treated with SIB had plasma GH similar to vehicle-treated mice on LF diet.

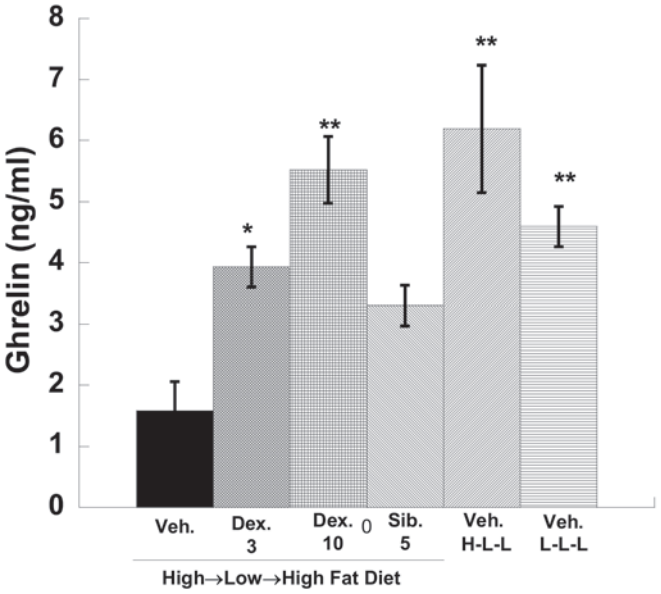

Fig. 2. Plasma ghrelin in diet-switched DIO mice. Treatment groups ($n = 12$/group): Veh, high–low–high vehicle-treated mice; DEX 3 mg/kg, po BID; DEX 10 mg/kg, po BID; SIB 5 mg/kg, po BID; Veh H-L-L, high low vehicle; Veh L-L-L, always LF vehicle. Statistically significant differences from Veh (H-L-H) group by one way analysis of variance, followed by Dunnett's post test: $^{**}p < 0.01$; $^{*}p < 0.05$ by Dunnett's test.

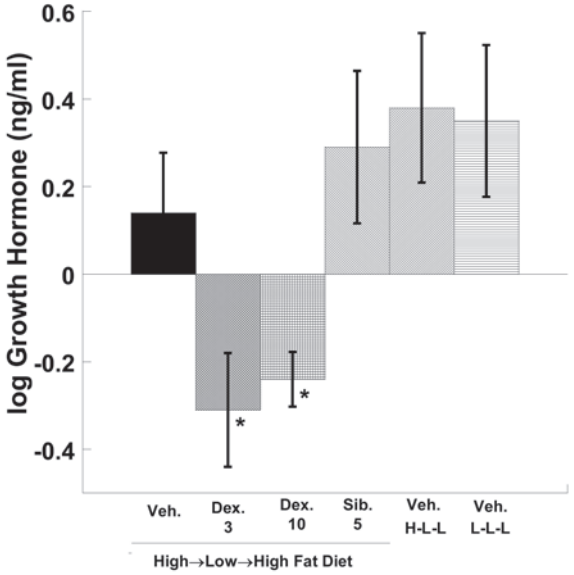

Fig. 3. Growth hormone in diet-switched DIO mice. Treatment groups ($n = 12$/group) same as noted in Fig. 3. $^{*}p < 0.05$ by Dunnett's test.

Vehicle-treated mice fed LF diet had significantly lower plasma insulin (Fig. 4) than mice fed HF diet ($p < 0.01$). Mice treated with DEX 10 mg/kg or 3 mg/kg had significantly decreased insulin relative to vehicle-treated DIO mice, while mice treated with SIB had hyperinsulinemia not significantly different from vehicle treated mice on HF diet.

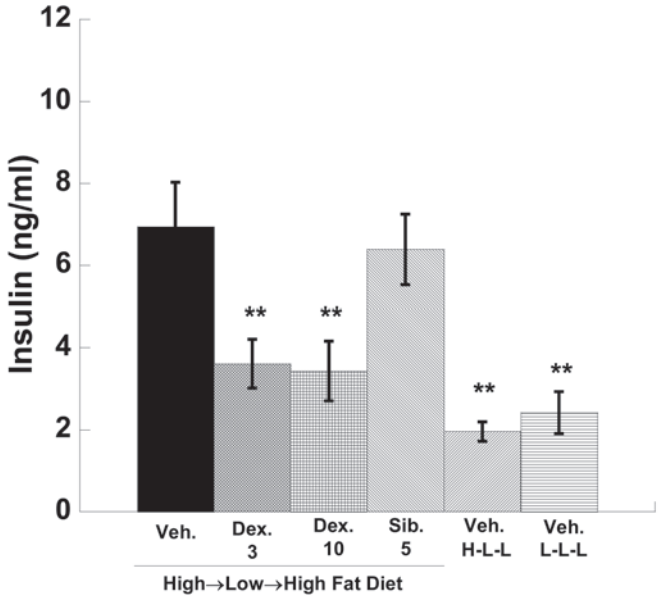

Fig. 4. Plasma insulin in diet-switched DIO mice. Treatment groups ($n = 12$/group) same as noted in Fig. 3. $^{**}p < 0.01$ by Dunnett's test.

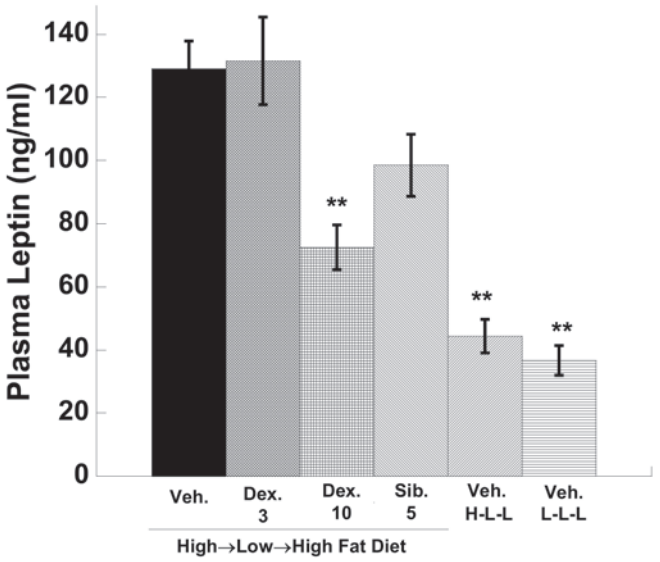

Fig. 5. Plasma leptin in diet-switched DIO mice. Treatment groups ($n = 12$/group) same as noted in Fig. 3. $^{**}p < 0.01$ by Dunnett's test.

Vehicle-treated mice fed LF diet had significantly lower plasma leptin (Fig. 5) than mice fed HF diet ($p < 0.01$). DEX 10 mg/kg significantly decreased plasma leptin. Leptin level in mice treated with SIB was not significantly different from vehicle treated mice on HF diet.

Vehicle-treated mice fed LF diet, either since weaning or since the first diet switch 50 d before study, had significantly lower EFP mass (Fig. 6) and total body fat mass determined by DEXA (Fig. 7A) than vehicle-treated mice fed HF diet. DEX 10 mg/kg significantly decreased both EFP mass and total fat mass. DEX 3 mg/kg and SIB did not affect EFP mass or total fat mass significantly. Lean body

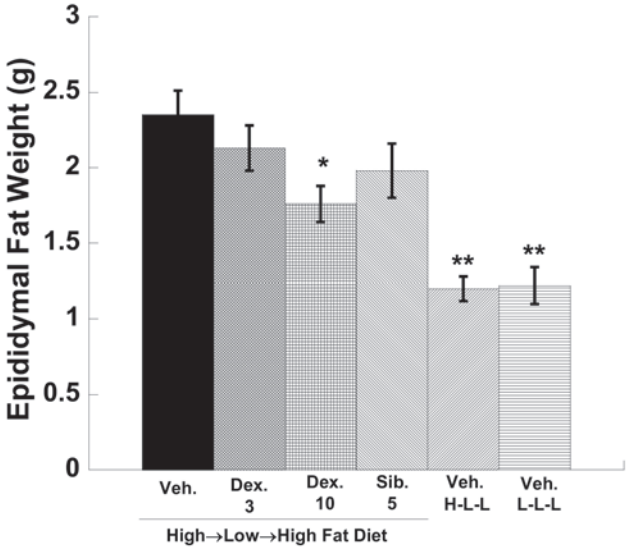

Fig. 6. Epididymal fat pad weight in diet-switched DIO mice. Treatment groups (n = 12/group) same as noted in Fig. 3. $^{**}p < 0.01$; $^{*}p < 0.05$ by Dunnett's test.

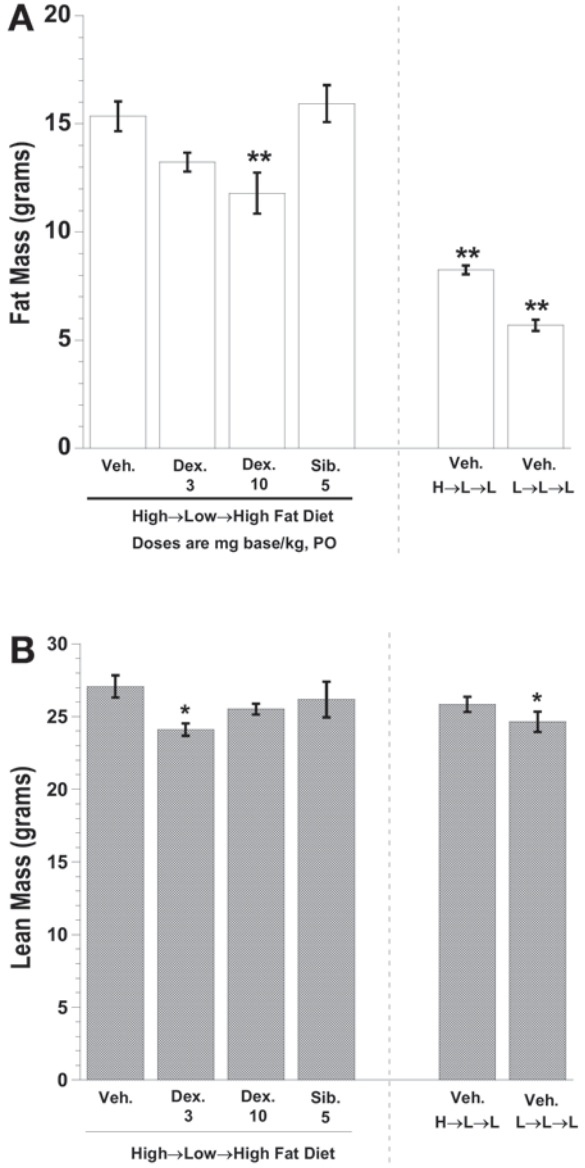

Fig. 7. Whole body fat mass and lean body mass by DEXA analysis in diet-switched DIO mice (n = 6/group), treatment groups same as noted in Fig. 3. (**A**) Fat mass, $^{**}p < 0.01$ by Dunnett's test. (**B**) Lean body mass, $^{*}p < 0.05$ by Dunnett's test.

mass measured by DEXA was significant lower in vehicle-treated mice always on LF diet ($p < 0.05$) than in vehicle treated mice fed HF diet. Lean mass was also significantly lower in mice treated with DEX 3 mg/kg ($p < 0.05$, Fig. 7B).

Discussion

The intent of the current study was to characterize the effects of dexfenfluramine (DEX) and sibutramine (SIB) on body weight gain and several hormones in mice that have lost weight due to caloric restraint. Individually housed DIO mice were converted to LF diet for 50 d. HF diet was reintroduced at the same time that 28-d treatments with DEX or SIB were initiated. Effects on body weight, food intake, plasma ghrelin, growth hormone, insulin, and leptin were observed.

C57BL/6J mice on a HF diet develop obesity, associated with chronic, excessive daily caloric intake. Mice on a HF diet consume more food (kcal) than mice on a LF diet. Reduction of dietary fat results in weight loss, and resumption of a HF diet results in a prompt weight regain *(6)*. In the current study, DEX (3 or 10 mg/kg) or SIB slowed weight gain significantly. Although either agent, relative to vehicle treatment slowed weight gain significantly, neither agent prevented weight gain over time in this diet-switched mouse model. Patients with obesity treated with SIB maintained weight loss for at least 2 yr, as demonstrated in the Storm Trials *(6)*. The results of the current study in mice yielded much less favorable results, SIB-treated mice gained weight over time although less than vehicle-treated mice. However, the DIO mice that remained on LF diet during treatment with vehicle failed to gain weight. In the Storm Trial, a 600 cal/d caloric deficit was maintained throughout the study, and 30 min of exercise per day was recommended. The mice in the current study were housed individually, were fed an unrestricted calorie-dense diet, and were not exercised, so the compounds were evaluated in the absence of any potentially beneficial lifestyle modifications.

The rate of caloric intake was decreased during the first 2 d of treatment with DEX at 3 or 10 mg/kg, or with SIB, relative to vehicle-treated mice on HF diet. Only DEX at the lower 3 mg/kg dose decreased caloric intake beyond 2 d of treatment, relative to HF-vehicle control mice. Vehicle-treated mice maintained on LF diet *ad libitum* had a lower voluntary caloric intake than mice did on HF diet, throughout most of the 28-d treatment period. Acute DEX treatment of rats at an appetite-suppressive dose increases Fos-like immunoreactivity in the arcuate nucleus, in pro-

opiomelanocortin (POMC) neurons, and the anorexic effects of DEX can be blocked by the melanocortin MC3/MC4 receptor antagonist SHU9119 administered in the third ventricle *(7)*. These results implicate the activation of hypothalamic POMC neurons in the anorexic effects of DEX. Treatment of diet-induced obese rats for 10 wk with SIB 5 mg/kg daily decreased the rate of food intake only for the first 3 wk, although body weight loss was maintained throughout the 10 wk *(8)*. Restriction of caloric intake to 90% of the *ad libitum* rate resulted in elevated NPY and decreased POMC mRNA expression in the arcuate nucleus after 10 wk, and these changes were significantly blunted by sibutramine treatment. Sibutramine also increased 24-h urinary norepinephrine excretion after 9 wk treatment, indicative of peripheral sympathetic activation *(8)*. Neither hypothalamic expression of POMC/NPY mRNA nor markers of peripheral energy expenditure were evaluated in the current study. The prolonged decrease in weight gain with only transient suppression of caloric intake suggests the slowing of weight gain cannot be attributed to a chronic anorexic effect, more likely it results from other factors, such as increased energy expenditure.

We have previous observed that switching DIO mice to *ad libitum* LF diet did not significantly increase plasma ghrelin over 28 d, although restriction of HF diet to 75% or less of *ad libitum* increased ghrelin significantly *(9)*. Others have demonstrated in human subjects that fat restriction for 2 wk avoids the increase in ghrelin levels caused by dietary energy restriction *(10)*. In the current experiment, mice had been switched back to HF diet after 50 d on LF diet. There was a difference in ghrelin levels at the end of study with significantly higher levels in the two groups of mice fed LF diet than in mice switched to HF diet. The mice switched to and remaining on LF diet were evaluated for ghrelin at 78 d after the switch from HF diet, a much longer interval than our previous study. It is possible that other factors than diet composition, such as differences in body weight, emerge as the primary long-term controller of ghrelin. Ghrelin levels are decreased in human obesity *(11)*. In the diet-switched DIO mice, DEX significantly and dose-dependently increased circulating ghrelin. SIB had no significant effect on end-of-study plasma ghrelin. The effects of DEX or SIB on circulating ghrelin have not been reported previously, and the mechanism for an increased ghrelin with DEX treatment is not clear. Although circulating ghrelin primarily is secreted from endocrine cells in the gastric mucosa, and there are also serotonin-containing enterochromaffin cells in the stomach *(12)*, there is no evidence of direct control of ghrelin secretion by serotonin. More likely, ghrelin levels are increased indirectly, possibly in response to the lesser weight gain in the DEX-treated mice. One additional factor to consider is the presence of ghrelin in hypothalamic neurons *(13)*, its role in energy balance, and modulation by dietary factors and pharmacotherapy. Presumably, increased circulating ghrelin may lead to increased appetite, thereby offsetting the direct effects of these agents on central control of energy balance.

Mice fed a HF diet will develop hyperinsulinemia, insulin resistance, and glucose intolerance. A significant decrease in circulating insulin with DEX treatment or continuation of a LF diet suggests that there is an improvement in whole body insulin sensitivity. DEX 3 or 10 mg/kg significantly lowered plasma insulin while the 10 mg/kg DEX treatment also decreased plasma leptin. SIB did not significantly alter plasma insulin or leptin. Studies in human subjects with type 2 diabetes have been conducted with fenfluramine in conjunction with phentermine *(14)* and with SIB monotherapy *(15)*, demonstrating improved glycemia and hemoglobin A1C. In the SIB study *(15)*, patients treated with SIB but not receiving insulin treatment had a decrease in insulin levels.

Human obese subjects have decreased plasma GH *(16)*, and increased GH and GHRH responsiveness *(17)* accompany weight loss in humans. DEX further decreases circulating GH in human obese subjects *(18)*, despite the weight loss. The functional consequence of this has not been demonstrated, but potentially loss of GH could lead to a decrease in lean body mass. In the current study, DEX 10 mg/kg, BID, significantly decreased GH ($p < 0.05$). GH release from the pituitary is stimulated by GHRH, originating from the arcuate nucleus *(19)*. GH release is also stimulated by ghrelin via GHS-R1 receptors in the arcuate and ventromedial nucleus *(20)*. The ghrelin signal originates from peripheral sources (stomach), as well as in the lateral hypothalamus. Because ghrelin was increased by DEX while GH was decreased, the decrease in GH is likely due to a ghrelin-independent effect, possibly a central serotoninergic inhibition of GHRH. In our study, HF diet fed mice treated with SIB had plasma GH similar to vehicle treated mice on LF diet, in agreement with the report that SIB had no effect on GHRH-stimulated GH secretion in rats *(21)*. Growth hormone (GH) is an important factor in the control of energy metabolism *(22,23)*. The physiological role of an increase in GH during weight loss is to stimulate the utilization of fat energy and inhibit the use of carbohydrates, thereby preventing hypoglycemia and defending lean body mass.

One shortcoming in the design of the current study is the reliance on terminal blood samples for characterization of hormone responses. Unfortunately it is difficult to obtain adequate quantities of blood for multiple analytes without euthanization, and serial large volume blood sampling is likely to affect body weight, food intake, and hormone profile. Because the effects of DEX and SIB on caloric intake are transient, it would be of interest to see if acute and transient changes in ghrelin, growth hormone, insulin, and leptin are temporally related with the loss of appetite and body weight changes. A further complication is the pulsatile nature of hormone secretion, particularly growth hormone, where single, discrete blood samples during the light phase of the light cycle at best show an average level at a single time of

day. Morning blood samples may not be optimal for the study of the physiology of metabolism in nocturnal animals. A more comprehensive analysis of the hormonal effects of DEX and SIB treatment would include multiple blood samples at several time points throughout the study, either in separate groups of mice for each time point or in a larger species than mice with serial blood sampling.

Effects of treatment on fat mass were assessed by wet weights of the epididymal fat pads and by whole body DEXA. Several different methods are available for evaluation of fat mass and lean body mass in mice. The gold standard method is carcass analysis, which requires desiccation to remove water content followed by extraction and chemical analysis. DEXA, or dual energy X-ray absorptiometry, is a method for measurement of tissue mass based on differential attenuation of high- and low-energy X-rays by tissues of different composition. Several studies have evaluated the precision and accuracy of DEXA for this purpose *(24,25)* using the GE-Lunar Piximus2 device. Both indicated that the calculation of body mass components using the manufacturer's software overestimates the fat mass, and a calibration procedure for correction based on comparison to carcass analysis is provided in the second paper *(25)*. The coefficient of variation for measurement of body fat content is 1.4% and body fat percentage is 1.2%.

Fat mass determined by either method was significantly lower in the two groups of mice receiving LF diet, than in the control mice on HF diet. In addition, fat mass was significantly lower in mice treated with DEX 10 mg/kg. DEX 3 mg/kg or SIB treatment did not significantly affect fat mass determined by either method. DEXA analysis also revealed a significantly lower lean body mass in the LF vehicle-treated groups compared to vehicle-treated HF mice. Mice treated with DEX 3 mg/kg also had significantly lower lean mass than vehicle-treated HF mice. In a 3 wk weight loss study in DIO rats, DEX at 3 and 10 mg/kg once a day and SIB at 3 mg/kg once a day decreased lean and fat mass as measured by magnetic resonance relaxometry *(26)*.

In summary, diet-switched DIO mice were used for the evaluation of the antiobesity agents DEX and SIB. Both compounds significantly delayed the regain of body weight associated with a switch from LF to HF diet. DEX increased circulating ghrelin, and decreased GH, leptin, insulin, and both whole body lean and fat mass, while SIB had no significant effect on these parameters in this model.

Materials and Methods

All in vivo experiments were conducted in accordance with guidelines established by Abbott Laboratories' Animal Care and Use Committee and National Institutes of Health Guide for Care and Use of Laboratory Animals. Male C57BL/6J mice (age 5–6 wk) were obtained from Jackson Labs (Bar Harbor, ME) and group housed in groups of five under conditions of 12 h lights on, 12 h lights off (on at 04:00 h), with food and water available *ad libitum*. At the beginning of the study, mice were administered a purified low-fat diet (LF, D12450Bi, 10 kcal% fat, 3.8 kcal/g) or a high-fat-content diet (HF, D12492i, 60 kcal% fat, 5.2 kcal/g), both obtained from Research Diets Inc. (New Brunswick, NJ) for 16 wk. The fat content of these diets was a mixture of lard and soybean oil.

Food intake monitoring was initiated at 22 wk of age and average 45 g body weight in the obese mice and 32 g in the leans. Fifty days before the beginning of treatment, DIO and lean mice were weighed, and individually housed in transparent plastic shoebox cages. Obese mice switched from HF diet to LF diet for 50 d, and mice to be continued on LF diet, were placed in clean cages and fresh bedding. Diet was switched back to HF, and treatment concurrently initiated for an additional 28 d.

Pharmacological treatments were administered twice a day (BID) at 08:00 h and 15:00 h. Mice were conditioned to oral gavage and daily vehicle administration for 1 wk prior to drug administration. There were six treatment groups (n = 12/group) in this study. Groups 1–5 were DIO mice switched to LF for 50 d. Groups 1–4 were switched back to HF diet for an additional 28 d, during which time they were treated with compounds or vehicle. Group 1 DIO mice received DEX 3 mg/kg by oral gavage (po) BID × 4 wk. Group 2 DIO mice received DEX 10 mg/kg po BID × 4 wk. Group 3 DIO mice were treated with SIB 5 mg/kg po BID × 4 wk. Group 4 DIO mice received vehicle 4 mL/kg BID × 4 wk (weight regain controls). Group 5 DIO mice remained on LF diet, and received vehicle po BID (weight loss controls). Group 6 mice were age-matched lean mice on LF throughout the study, treated with vehicle during the final 4 wk BID (lean controls with treatment). All compound doses are expressed as base equivalent weights per unit body weight. The vehicle used for conditioning and study was 1% Tween-80 (Sigma Chemical, St. Louis, MO) in water. All doses were given in 4 mL vehicle per kg body weight. Food weights and body weights were determined on d 0, 1, 3, 5, 8, 14, 20, and 28.

The higher dose of dexfenfluramine (10 mg/kg) was used in a previous weight loss study in DIO mice *(27)*. The dose of sibutramine selected (5 mg/kg) is based on a published chronic antiobesity study *(28)*, in which *ob/ob* mice treated once a day for 6 wk had a significant decrease in rate of weight gain and a 31% decrease in plasma insulin.

On d 28, mice were humanely euthanized under CO_2–O_2 anesthesia, starting at 8:30 AM. A terminal blood sample obtained by cardiac puncture was placed in a tube containing EDTA anticoagulant, mixed by inversion, and maintained at 4°C until centrifugation at 1400*g* for 10 min. Plasma was stored at –80°C until assay. Body composition (lean and fat mass) was determined after decapitation in a subgroup of six mice per treatment group by DEXA (Pixi-Mus2, GE

Lunar Corp, Madison WI), using software version 1.46. Fat and lean masses were corrected according to the published calibration *(25)*. Epididymal fat pads were removed to obtain tissue weights. Plasma samples were assayed for insulin (Alpco Diagnostics, Windham NH) and leptin (Crystalchem, Downers Grove, IL) by ELISA, and GH and total ghrelin (Linco Research, Inc, St. Charles MO) by RIA. The sensitivities, intraassay and interassay coefficients of variation for these assays are as follows: insulin 0.07 ng/mL, 3.3%, 1.8%; leptin 0.2 ng/mL, 5.4% and 6.9%; ghrelin 0.093 ng/mL, 6.4% and 16.3%; GH 0.5 ng/mL, 5.6% and 8.7%.

Body weight change and caloric intake data over time were analyzed by two-way analysis of variance with repeated measures, using GraphPad Prism 4 for Windows (GraphPad, San Diego, CA). Where significant differences among treatments and time points were detected, a Bonferroni's post hoc test was used for comparisons to the vehicle-treated high-fat group (HF–LF–HF). Statistical analyses of terminal plasma analytes, DEXA, and tissue weight data were performed by one-way analysis of variance, and, if significant, followed by Dunnett's test, using GraphPad InStat (Graphpad, San Diego, CA). Differences were considered significant where $p < 0.05$. Data are presented as mean ± SEM.

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